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CA INDEXING COPYRIGHT (C) 2000 AMERICAN CHEMICAL SOCIETY (ACS)

=> s antibody (p) humanized (p) fusion (p) interleukin

L1 18 ANTIBODY (P) HUMANIZED (P) FUSION (P) INTERLEUKIN

=> dup rem l1

PROCESSING COMPLETED FOR L1

L2 10 DUP REM L1 (8 DUPLICATES REMOVED)

=> d l2 total ibib kwic

L2 ANSWER 1 OF 10 USPATFULL

ACCESSION NUMBER: 2000:41155 USPATFULL

TITLE: FAS ligand fusion proteins and their uses

INVENTOR(S): Queen, Cary L., Los Altos, CA, United States
Schneider, William P., Los Altos, CA, United States
Vasquez, Maximiliano, Palo Alto, CA, United States

PATENT ASSIGNEE(S): Protein Design Labs., Inc., Fremont, CA, United States
(U.S. corporation)

	NUMBER	DATE
PATENT INFORMATION:	US 6046310	20000404
APPLICATION INFO.:	US 1997-815190	19970311 (8)
RELATED APPLN. INFO.:	Continuation-in-part of Ser. No. US 1996-614584, filed on 13 Mar 1996, now abandoned	
DOCUMENT TYPE:	Utility	
PRIMARY EXAMINER:	Chan, Christina Y.	

ASSISTANT EXAMINER: Nolan, Patrick J.
LEGAL REPRESENTATIVE: Townsend & Townsend & Crew LLP
NUMBER OF CLAIMS: 8
EXEMPLARY CLAIM: 1
NUMBER OF DRAWINGS: 6 Drawing Figure(s); 7 Drawing Page(s)
LINE COUNT: 1454

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

DETD In one aspect, the present invention is directed to a **fusion** protein comprising a functional moiety of the extracellular domain of the FasL protein and a polypeptide capable of specifically binding. . . antigen, expressed on particular cell-type(s) or tissue(s). In a preferred embodiment, the binding polypeptide comprises the variable domain of an **antibody**. In a particularly preferred embodiment, the **antibody** is **humanized**, human or from another primate species. However, the binding polypeptide may also comprise, for example, the binding site of a cellular receptor; a receptor ligand such as a cytokine, lymphokine, **interleukin**, growth factor, hormone or the like; or the binding site of an adhesion molecule, such as a selectin or integrin.. . . region (e.g., an integral membrane protein or a transmembrane glycoprotein), wherein said extracellular portion can be specifically bound by an **antibody** or other ligand, i.e., with an affinity of stronger than about 1.times.10.sup.6 M.sup.-1. The term cell surface marker also refers. . . specificity of an intact FasL polypeptide, but will be soluble rather than membrane bound. Preferably, the FasL component of the **fusion** protein is found within a segment of up to about 10, 25 or 50 amino acids within the FasL extracellular. . .

L2 ANSWER 2 OF 10 BIOSIS COPYRIGHT 2000 BIOSIS

ACCESSION NUMBER: 2000:244980 BIOSIS

DOCUMENT NUMBER: PREV200000244980

TITLE: In vivo anti-tumor activity of an anti-HER-2/neu-IL-12 antibody fusion protein.

AUTHOR(S): Peng, Lisan S. (1); Penichet, Manuel L. (1); Morrison, Sherie L. (1)

CORPORATE SOURCE: (1) UCLA, Los Angeles, CA USA

SOURCE: Proceedings of the American Association for Cancer Research

Annual Meeting, (March, 2000) No. 41, pp. 287.

Meeting Info.: 91st Annual Meeting of the American Association for Cancer Research. San Francisco,

California,

USA April 01-05, 2000

ISSN: 0197-016X.

DOCUMENT TYPE: Conference

LANGUAGE: English

SUMMARY LANGUAGE: English

IT . . .

Diseases

breast cancer: neoplastic disease, reproductive system disease/female;

ovarian cancer: neoplastic disease, reproductive system disease/female

IT Chemicals & Biochemicals

anti-HER-2/neu-IL-12 **antibody fusion** protein

[mscIL-12.her2.IgG3]: antineoplastic - drug, immunologic - drug, in vitro activity; **humanized** anti-HER-2/neu: antineoplastic -

drug; **interleukin-12**; tumor-specific **antibodies**

IT Alternate Indexing

Breast Neoplasms (MeSH); Ovarian Neoplasms (MeSH)

L2 ANSWER 3 OF 10 USPATFULL

ACCESSION NUMBER: 1999:146362 USPATFULL

TITLE: Antisense inhibition of interleukin-15 expression
INVENTOR(S): Bennett, C. Frank, Carlsbad, CA, United States
Cowser, Lex M., Carlsbad, CA, United States
PATENT ASSIGNEE(S): Isis Pharmaceuticals Inc., Carlsbad, CA, United States
(U.S. corporation)

	NUMBER	DATE
PATENT INFORMATION:	US 5985663	19991116
APPLICATION INFO.:	US 1998-200141	19981125 (9)
DOCUMENT TYPE:	Utility	
PRIMARY EXAMINER:	LeGuyader, John L.	
ASSISTANT EXAMINER:	Schmidt, Melissa	
LEGAL REPRESENTATIVE:	Law Offices of Jane Massey Licata	
NUMBER OF CLAIMS:	19	
EXEMPLARY CLAIM:	1	
LINE COUNT:	2790	

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

SUMM One monoclonal **antibody** to **Interleukin-15**, M110, has been used to inhibit the upregulation of natural killer (NK) and **antibody**-dependent cellular cytotoxicity (ADCC) activities in the peripheral blood mononuclear cells of HIV-sero-negative patients (Loubeau et al., J. Acquir. Immune Defic. Syndr. Hum. Retrovirol., 1997, 16, 137-145). Likewise, another commercially available **antibody**, Mill, was used to demonstrate that **Interleukin-15** is involved in the regulation of markers of melanoma progression (Barzegar et al., Oncogene, 1998, 16, 2503-2512). Furthermore, **humanized** and **bispecific-humanized antibodies** to the IL-2 receptor .alpha. and .beta. subunits have been shown to reduce the **Interleukin-15**-induced proliferation of several cell types (Guex-Crosier et al., J. Immunol., 1997, 158, 452-458; Pilson et al., J. Immunol., 1997, 159, 1543-1556). Finally, using site-directed mutagenesis, Kim et al. produced an **InterLeukin-15** antagonist, a mutant **Interleukin-15**/murine Fcgamma2a **fusion** protein, which inhibited **Interleukin-15**-triggered cell proliferation in BAF-BO3 cells and blocked delayed-type sensitivity in mice (Kim et al., J. Immunol., 1998, 160, 5742-5748). However, . . .

L2 ANSWER 4 OF 10 EMBASE COPYRIGHT 2000 ELSEVIER SCI. B.V.DUPLICATE 1

ACCESSION NUMBER: 1999287254 EMBASE

TITLE: Preparation and characterization of a recombinant **humanized** single- chain Fv **antibody**/human **interleukin-2 fusion** protein directed against the HER-2/neu (c-erbB2) proto-oncogene product, p185.

AUTHOR: Li J.; Gyorffy S.F.; Ring D.B.; Kwok C.S.; Austin R.C.
CORPORATE SOURCE: R.C. Austin, Hamilton Civic Hospitals Res. Centre, 711 Concession St., Hamilton, Ont. L8V 1C3, Canada

SOURCE: Tumor Targeting, (1999) 4/2 (105-114).

Refs: 35

ISSN: 1351-8488 CODEN: TUTAF

COUNTRY: United Kingdom

DOCUMENT TYPE: Journal; Article

FILE SEGMENT: 016 Cancer
026 Immunology, Serology and Transplantation
037 Drug Literature Index

LANGUAGE: English

SUMMARY LANGUAGE: English

TI Preparation and characterization of a recombinant **humanized** single- chain Fv **antibody**/human **interleukin-2 fusion** protein directed against the HER-2/neu (c-erbB2) proto-oncogene product, p185.

AB High dose recombinant human **interleukin 2** (rhIL-2) therapy has been used in the treatment of established tumors in both animal models and

patients with advanced. . . because high dose rhIL-2 therapy causes severe systemic toxicity in normal tissues, its clinical use has been limited. Therefore, targeting **interleukin-2** (IL-2) to the tumor site should improve its anti-tumor-immune response and decrease its systemic toxicity. In this study, we describe the preparation and characterization of a recombinant **humanized** single-chain Fv (sFv) **antibody/IL-2 fusion** protein. This recombinant **fusion** protein consists of **humanized** variable heavy (V(H)) and light (V(L)) domains of monoclonal **antibody** (mAb) 520C9 directed against the human HER-2/neu(c-erbB2) proto-oncogene product p185 and human IL-2. The **fusion** protein was stably expressed in baby hamster kidney cells and shown to retain the immunostimulatory activities of IL-2 as measured by IL-2-dependent cell proliferation and cytotoxicity assays. In addition to its IL-2 activity, this **fusion** protein also possesses binding specificity against the HER-2/neu (c-erbB2) proto-oncogene product, p185, as determined by enzyme linked immunosorbent assay (ELISA) using SKOV 3ip1 cells. Taken together, these findings suggest that this recombinant **humanized sFv antibody /IL-2 fusion** protein may provide an effective means of targeting therapeutic doses of IL- 2 to p185 positive tumors without increasing systemic. . .

L2 ANSWER 5 OF 10 BIOSIS COPYRIGHT 2000 BIOSIS
 ACCESSION NUMBER: 1998:438453 BIOSIS
 DOCUMENT NUMBER: PREV199800438453
 TITLE: Humanized monoclonals and other biological initiatives.
 AUTHOR(S): Halloran, Philip F. (1); Prommool, Surazee
 CORPORATE SOURCE: (1) Univ. Alberta, Div. Nephrol. Immunol., Dep. Med. 303, 8249-114 St., Edmonton, AB T6G 2R8 Canada
 SOURCE: Clinical Biochemistry, (July, 1998) Vol. 31, No. 5, pp. 353-357.
 ISSN: 0009-9120.
 DOCUMENT TYPE: General Review
 LANGUAGE: English
 IT Major Concepts
 Immune System (Chemical Coordination and Homeostasis); Pharmacology
 IT Chemicals & Biochemicals
 anti-**interleukin-2** receptor monoclonal **antibodies**;
 anti-CD40L; **fusions** proteins; **humanized**
 monoclonals: immunosuppressant effect; CTLA4Ig

L2 ANSWER 6 OF 10 MEDLINE DUPLICATE 2
 ACCESSION NUMBER: 1998014585 MEDLINE
 DOCUMENT NUMBER: 98014585
 TITLE: Elimination of established murine colon carcinoma metastases by antibody-interleukin 2 fusion protein therapy.
 AUTHOR: Xiang R; Lode H N; Dolman C S; Dreier T; Varki N M; Qian X;
 Lo K M; Lan Y; Super M; Gillies S D; Reisfeld R A
 CORPORATE SOURCE: Department of Immunology, The Scripps Research Institute, La Jolla, California 92037, USA.
 CONTRACT NUMBER: CA42508 (NCI)
 SOURCE: CANCER RESEARCH, (1997 Nov 1) 57 (21) 4948-55.
 Journal code: CNF. ISSN: 0008-5472.
 PUB. COUNTRY: United States
 Journal; Article; (JOURNAL ARTICLE)
 LANGUAGE: English
 FILE SEGMENT: Priority Journals; Cancer Journals
 ENTRY MONTH: 199801
 ENTRY WEEK: 19980104
 AB A recombinant **humanized antibody-interleukin**
 2 **fusion** protein (huKS1/4-IL-2) was used to direct IL-2 to the

tumor microenvironment and elicit a T cell-mediated eradication of established pulmonary and hepatic CT26-KSA colon carcinoma metastases in syngeneic BALB/c mice. This antitumor effect was specific because a **fusion** protein, which was nonreactive with these tumor cells, failed to exert any such effect. The efficacy of the huKS1/4-IL-2 **fusion** protein in eliminating metastases was documented because mixtures of monoclonal **antibody** huKS1/4 with recombinant human IL-2 were ineffective and, at best, only partially reduced tumor load.

Two

lines of evidence indicated the eradication of metastases and the absence of minimal residual disease in animals treated with the **fusion** protein: first, the lack of detection of CT26-KSA cells by reverse transcription-PCR, which can detect one tumor cell in 10(6). . . were further characterized as CD8+ T cells by in vivo depletion studies. Such

T

cells, isolated from tumor-bearing mice after **fusion** protein therapy, elicited MHC class I-restricted cytotoxicity in vitro against colon carcinoma target cells. Taken together, these data indicate that **fusion** protein-directed IL-2 therapy induces a T cell-dependent host immune response capable of eradicating established colon cancer metastases in an animal. . .

L2 ANSWER 7 OF 10 MEDLINE DUPLICATE 3
ACCESSION NUMBER: 93342357 MEDLINE
DOCUMENT NUMBER: 93342357
TITLE: [Anti-cytokines and anti-cytokine receptors].
Anticytokines et antirecepteurs de cytokine.
AUTHOR: Dantal J; Giral M; Soullillou J P
CORPORATE SOURCE: INSERM U211 Unite de recherche sur les effecteurs
lymphocytaires T, Plateau technique, CHU Hotel-Dieu,
Nantes..
SOURCE: REVUE DU PRATICIEN, (1993 Mar 1) 43 (5) 586-9. Ref: 18
Journal code: T1D. ISSN: 0035-2640.
PUB. COUNTRY: France
Journal; Article; (JOURNAL ARTICLE)
General Review; (REVIEW)
(REVIEW, TUTORIAL)
LANGUAGE: French
FILE SEGMENT: Foreign
ENTRY MONTH: 199311
AB Cytokines play a key-role in the immune response. The best known of them
is **interleukin-2** and its specific receptors. Monoclonal
antibodies directed against the **interleukin-2** receptor
have initially enabled this receptor to be characterized; then they
served
to confirm the major role played by this. . . in man, particularly in
kidney transplantation (but also in bone marrow transplantation), and
they
encourage to develop new bioreagents (chimeral **antibodies**, "
humanized" **antibodies**, **fusion** proteins). Some
of these reagents are now undergoing evaluation in renal transplantation.
The principles of these bioreagents, issued from molecular. . .

L2 ANSWER 8 OF 10 CAPLUS COPYRIGHT 2000 ACS
ACCESSION NUMBER: 1994:649853 CAPLUS
DOCUMENT NUMBER: 121:249853
TITLE: Receptor-affinity chromatography (RAC)
AUTHOR(S): Nachman-Clewner, Michele; Spence, Cheryl; Bailon,
Pascal
CORPORATE SOURCE: Dep. Physiol., Cornell Med. Sch., New York, NY,
10021,
USA
SOURCE: Mol. Interact. Biosep. (1993), 139-49. Plenum
CODEN: 60ESAG
DOCUMENT TYPE: Conference; General Review
LANGUAGE: English

AB A review with many refs. describing the use of the title method for the purifn. of recombinant human **interleukin-2** (rIL-2) rIL-2 muteins, murine rIL-2, an **interleukin-2** Pseudomonas exotoxin **fusion** protein (IL2-PE40), and a **humanized** monoclonal **antibody** to IL-2 receptor using a multipurpose IL-2R affinity adsorbent.

L2 ANSWER 9 OF 10 MEDLINE DUPLICATE 4
ACCESSION NUMBER: 92388287 MEDLINE
DOCUMENT NUMBER: 92388287
TITLE: Membrane-based receptor affinity chromatography.
AUTHOR: Nachman M; Azad A R; Bailon P
CORPORATE SOURCE: Protein Biochemistry Department, Roche Research Center, Hoffmann-La Roche Inc., Nutley, NJ 07110..
SOURCE: JOURNAL OF CHROMATOGRAPHY, (1992 Apr 24) 597 (1-2) 155-66.
Journal code: HQF. ISSN: 0021-9673.
PUB. COUNTRY: Netherlands
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199212

AB . . . the molecular recognition between an immobilized receptor and its soluble protein ligand, has been developed for the purification of human **interleukin-2** and related biomolecules. The multi-purpose affinity membrane used in this study consisted of a soluble form of **interleukin-2** receptor (IL-2R) chemically bonded to hollow-fiber membranes in an oriented fashion. A model system involving anti-Tac-H (a **humanized** monoclonal **antibody** to IL-2R) was used to study the important factors influencing the performance of MRAC, including support morphology, mass transfer rate. . . All three are shown to be highly efficient. MRAC has been successfully applied to the purification of anti-Tac-H, recombinant human **interleukin-2** (rIL-2) and **interleukin 2**-Pseudomonas exotoxin **fusion** protein (IL2-PE40). Overall, MRAC was found to be a viable, scalable and extremely productive affinity purification method.

L2 ANSWER 10 OF 10 MEDLINE DUPLICATE 5
ACCESSION NUMBER: 91185530 MEDLINE
DOCUMENT NUMBER: 91185530
TITLE: Lymphokine receptor-directed therapy: a model of immune intervention.
AUTHOR: Waldmann T A; Grant A; Tendler C; Greenberg S; Goldman C; Bamford R; Junghans R P; Nelson D
CORPORATE SOURCE: Metabolism Branch, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892..
SOURCE: JOURNAL OF CLINICAL IMMUNOLOGY, (1990 Nov) 10 (6 Suppl) 19S-28S; discussion 28S-29S. Ref: 57
Journal code: HRC. ISSN: 0271-9142.
PUB. COUNTRY: United States
Journal; Article; (JOURNAL ARTICLE)
General Review; (REVIEW)
(REVIEW, TUTORIAL)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199107

AB We have proposed a multichain model for the high-affinity **interleukin-2** (IL-2) receptor involving two IL-2-binding peptides, a 70/75 kilodalton (kD) and a 55 kD, reactive with the anti-Tac monoclonal **antibody**, which are associated in a receptor complex. With the use of coprecipitation analysis, radiolabeled **interleukin-2** cross-linking procedures, and flow cytometric resonance energy transfer measurements, a series of additional peptides of molecular weight 22,000,

35,000, 40,000, 75,000 (non-IL-2 binding), 95,000-105,000, and 180,000 has been associated with the two **interleukin-2-binding** peptides. In contrast to resting T cells, the abnormal T cells of patients with human T-cell lymphotropic virus I-associated adult. . . in Tac antigen expression, we have initiated therapeutic trials using unmodified anti-Tac, conjugates of anti-Tac with truncated *Pseudomonas* exotoxin PE-40, **interleukin-2-truncated** toxin **fusion** proteins, and alpha- and beta-emitting isotopic chelates of anti-Tac. Furthermore, by genetic engineering **humanized** hyperchimeric anti-Tac molecules have been prepared in which the molecule is entirely human IgG1, except for the small complementarity-determining regions that are retained from the mouse **antibody**. This "**humanized**" **antibody** manifested the ability to perform **antibody** -dependent cellular cytotoxicity absent in the original mouse monoclonal. The clinical application of anti-**interleukin-2** receptor-directed therapy represents a new perspective for the treatment of certain neoplastic diseases and autoimmune disorders and for the prevention. .

=> s antibody (p) humanized (p) tnf

L3 90 ANTIBODY (P) HUMANIZED (P) TNF

=> s antibody (p) humanized (p) tnf (p) fusion

L4 11 ANTIBODY (P) HUMANIZED (P) TNF (P) FUSION

=> dup rem l4

PROCESSING COMPLETED FOR L4

L5 7 DUP REM L4 (4 DUPLICATES REMOVED)

=> d l5 total ibib kwic

L5 ANSWER 1 OF 7 USPATFULL

ACCESSION NUMBER: 2000:95042 USPATFULL

TITLE: Therapeutic methods employing disulfide derivatives of dithiocarbamates and compositions useful therefor

INVENTOR(S): Lai, Ching-San, Encinitas, CA, United States
Vassilev, Vassil, San Diego, CA, United States

PATENT ASSIGNEE(S): Medinox Inc., San Diego, CA, United States (U.S. corporation)

	NUMBER	DATE
PATENT INFORMATION:	US 6093743	20000725
APPLICATION INFO.:	US 1998-103639	19980623 (9)
DOCUMENT TYPE:	Utility	
PRIMARY EXAMINER:	Weddington, Kevin E.	
LEGAL REPRESENTATIVE:	Gary Cary Ware & Freidenrich; Reiter, Stephen E.; Kirschenbaum, Shelia R.	
NUMBER OF CLAIMS:	51	
EXEMPLARY CLAIM:	1	
NUMBER OF DRAWINGS:	11 Drawing Figure(s); 5 Drawing Page(s)	
LINE COUNT:	2691	

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

DETD . . . the primary treating agent include administration of agents for

the treatment of multiple sclerosis, such as 4-aminopyridine, deoxyspergualin, ACTH, amantadine, **antibody** adjuvants (e.g., poly-ICLC), anti-cytokine monoclonal **antibodies**,

anti-inflammatory agents, baclofen, bethanechol chloride, carbamazepine, carbohydrate drugs, clonazepam, CNS and immune system function modulators, cyclophosphamide, cyclosporine A, cytokines (e.g., . . . (e.g., AnergiX), CD28 antagonists, growth factors (e.g., glial growth factor, GGF, nerve growth factors, TGF- β 2, PEG-TGF- β 2, betakine, and the like), **humanized MAb** (e.g., anti-IFN- γ .MAb, smart anti-IFN- γ .MAb, anti-Tac **antibody**, smart anti-Tac **antibody**, and the like), **humanized anti-CD4 MAb** (e.g., anti-CD4 MAb, centara, and the like), hydrolase stimulants (e.g., castanospermine), IFN- α , IFN- γ antagonists (e.g., anti-IFN- γ .MAb, smart anti-IFN- γ .MAb, and the like), IL-2 antagonists (e.g., tacrolimus, Fujimycin, Prograf, IL-2 **fusion** toxin, DAB.sub.389 IL-2, and the like), IL-4 antagonists (e.g., IL-4 **fusion** toxin, DAB.sub.389 IL-4, and the like), immune-mediated neuronal damage inhibitors, immunoglobulins, immunostimulants (e.g., poly-ICLC, edelfosine, ET-18- OCH-3, ET-18-OME, and the like), immunosuppressants (e.g., azathioprine, castanospermine, tacrolimus, FK-506, Fujimycin, Prograf, anti-leukointegrin MAb, primatized anti-CD4 **antibody**, linomide, roquinimex, transcyclo-pentanyl purine analogs, spanidin, 15-deoxyspergualin, deoxyspurgiline, gusperimus HCl, cyclosporine, Sandimmune, IL-10, anti-TCR MAbs, anti-CD4 MAb, cantara, immunophilins, cyclophosphamide, and the like), integrin antagonists (e.g., anti-integrin monoclonal **antibodies**), interferon agonists, interferon- β -1b, isoprinosine, IV methylprednisolone, macrolides, MAO B inhibitors (e.g., selegiline, Parkinyl, and the like), methotrexate, mitoxantrone, muscarinic antagonists, . . . analogs (e.g., spanidin, 15-deoxyspergualin, deoxyspurgiline, gusperimus HCl, and the like), selectin antagonists (e.g., lectin-1, recombinant IML-1, and the like), soluble **TNF** receptor I, **TNF** antagonists (e.g., thalidomide, **TNF** inhibitors, and the like), and the like.

DETD . . . primary treating agent include administration of agents for the

treatment of systemic lupus erythematosus (SLE), such as androgen-derived steroids, anti-CD4 **humanized antibodies**, CD2 antagonists, cyclosporines (e.g., Sandimmune, cyclosporine analog, cyclosporin-G, NVal-CyA, and the like), cytokines (e.g., IL-4 **fusion** toxin), cytokine receptor antagonists (e.g., immunomodulatory cytokines), E-selectin antagonists (e.g.,

anti-E

LAM), FK506/tacrolimus (e.g., Prograf), hypercalcemic agents, IFN- γ antagonists (e.g., . . . the like), immunoglobulins

(e.g.,

anti-ELAM), immunostimulants (e.g., thymotrinan), immunosuppressants (e.g., Rapamycin, anti-CD4, T-cell inhibitor, anti-tac MAb, immunophilins, mycophenolate mofetil, IL-4 **fusion** toxin, trypanosomal inhibitory factor (TIF), Leflunomide, Spanidin, 15-deoxyspergualin, deoxyspurgiline, gusperimus hydrochloride, Roquinimex, linomide, and the like), immunotoxins (e.g., Zolomomab aritox, Xomazyme-CD5 Plus, and the like), intravenous immunoglobulins, integrin antagonists (e.g., integrin blockers), Migis.TM.

antibodies, monoclonal **antibody** therapeutics, murine MAb (e.g., anti-SLE vaccine, MAb 3E10, and the like), primatized anti-CD4 **antibodies** (e.g., CE9.1), protease inhibitors (e.g., matrix metalloprotease (MMP) inhibitors, stromelysin, and the like), protein synthesis antagonists (e.g., anti-CD6-bR, anti-T12-bR, oncolysin. . . Cylexin), spergualin analogues (e.g., Spanidin, 15-deoxyspergualin, deoxyspurgiline, gusperimus hydrochloride, and the like), T cell inhibitors (e.g., AnergiX), tumor necrosis factor (**TNF**) antagonists, and the like.

TITLE: Methods of treating TNF.alpha.-mediated disease using
chimeric anti-TNF antibodies
INVENTOR(S): Le, Junming; Vilcek, Jan; Dadonna, Peter; Ghrayeb,
John; Knight, David; Seigal, Scott
PATENT ASSIGNEE(S): New York University, USA; Centocor, Inc.
SOURCE: U.S., 88 pp., Cont.-in-part of U.S. Ser. No. 10,406,
abandoned.
CODEN: USXXAM
DOCUMENT TYPE: Patent
LANGUAGE: English
FAMILY ACC. NUM. COUNT: 4
PATENT INFORMATION:

PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
US 5919452	A	19990706	US 1994-192861	19940204
US 5698195	A	19971216	US 1994-324799	19941018
PRIORITY APPLN. INFO.:			US 1991-670827	19910318
			US 1992-853606	19920318
			US 1992-943852	19920911
			US 1993-10406	19930129
			US 1993-13413	19930202
			US 1994-192093	19940204
			US 1994-192102	19940204
			US 1994-192861	19940204

REFERENCE COUNT: 82
REFERENCE(S): (2) Aggarwal, B; J of Biol Chem 1985, V260(4), P2345
CAPLUS
(3) Akama, H; Biochemical and Biophysical Research
Comm 1990, V168(2), P857 CAPLUS
(4) Anon; EP 0212489 A2 1987 CAPLUS
(5) Anon; EP 0218868 A2 1987 CAPLUS
(7) Anon; EP 0288088 A2 1988 CAPLUS
ALL CITATIONS AVAILABLE IN THE RE FORMAT

IT **Fusion** proteins (chimeric proteins)
RL: BPN (Biosynthetic preparation); PRP (Properties); THU (Therapeutic
use); BIOL (Biological study); PREP (Preparation); USES (Uses)
(**TNF** receptor or anti-**TNF** antibody;
humanized rodent monoclonal IgG1 specific to human tumor
necrosis factor .alpha. or .beta. for treating infections and
autoimmune diseases)

L5 ANSWER 3 OF 7 USPATFULL

ACCESSION NUMBER: 1999:72602 USPATFULL
TITLE: Conjugates of dithiocarbamates with pharmacologically
active agents and uses therefore
INVENTOR(S): Lai, Ching-San, Encinitas, CA, United States
PATENT ASSIGNEE(S): Medinox, Inc., San Diego, CA, United States (U.S.
corporation)

	NUMBER	DATE
PATENT INFORMATION:	US 5916910	19990629
APPLICATION INFO.:	US 1997-869158	19970604 (8)
DOCUMENT TYPE:	Utility	
PRIMARY EXAMINER:	Davis, Zinna Northington	
LEGAL REPRESENTATIVE:	Reiter, Esq., Stephen E.Gray, Cary, Ware & Freidenrich	
NUMBER OF CLAIMS:	27	
EXEMPLARY CLAIM:	1	
LINE COUNT:	1842	

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

SUMM multiple sclerosis agents, such as 4-aminopyridine, 15.+-
.deoxyspergualin, ACTH, amantadine, **antibody** adjuvants (e.g.,
poly-ICLC, and poly-IC+poly-L-lysine+carboxymethylcellulose),
anti-cytokine MAb (CDP-835), anti-inflammatory (e.g., CY-1787, and
CY-1503), anti-selectin MAb (e.g., CY-1787), anti-TCR MAb (e.g., . . .

therapies (e.g., benzoporphyrin derivative (BPD)), FK-506, growth factors (e.g., glial growth factor, GGF, nerve growth factors, TGF- β 2, PEG-TGF- β 2, and betakine), **humanized MAb** (e.g., anti-IFN- γ .MAb, smart anti-IFN- γ .MAb, anti-Tac **antibody**, and smart anti-Tac **antibody**), **humanized anti-CD4 MAb** (e.g., anti-CD4 MAb, centara), hydrolase stimulants (e.g., castanospermine), IFN- α , IFN- γ . antagonist (e.g., anti-IFN- γ . MAb, and smart anti-IFN- γ . MAb), IL-2 antagonists (e.g., tacrolimus, FK-506, FR-900506, Fujimycin, Prograf, IL-2 **fusion** toxin, and DAB.sub.389 IL-2), IL-4 antagonists (e.g., IL-4 **fusion** toxin, and DAB.sub.389 IL-4), immune-mediated neuronal damage inhibitors (e.g., NBI-114, NBI-115, and NBI-116), immunoglobins, immunostimulants (e.g., poly-ICLC, edelfosine, ALP, ET-18-OCH₃, . . . AI-100 animal protein, rDNA human protein AI-101, peptide, AI-102, castanospermine, tacrolimus, FK-506, FR-900506, Fujimycin, Prograf, anti-leukointegrin MAb, Hu23F2G, primatized anti-CD4 **antibody**, CE9.1, Galaptin 14-1, GL14-1, Lectin-1, recombinant IML-1, linomide, roquinimex, LS-2616, transcyclo-pentanyl purine analogs, MS-6044, spanidin, 15-deoxyspergualin, deoxyspurgiline, gusperimus HCL, NSC-356894, . . . (e.g., NBI-114, NBI-115, and NBI-116), TCR peptide vaccines (e.g., AI-208 (V. β 6.2/6.5 phenotype)), selectin antagonists (e.g., lectin-1, and recombinant IML-1), soluble **TNF** receptor I, TCARs (e.g., TCR, CD3/Ti, and peptigen TP12), **TNF** antagonists (e.g., thalidomide, and **TNF** inhibitors), tricyclic antidepressants, and the like;

SUMM systemic lupus erythematosus (SLE) agents, such as androgen-derived steroids (e.g., Org-4094), anti-CD4 **humanized antibodies**, anti-DNA/V-88, anti-idiotypic murine MAb (e.g., anti-idiotypic **antibody** to 3E10/MAb1C7), CD2 antagonists (e.g., CD2), complement inhibitors (e.g., recombinant MCP-based complement inhibitors), cyclosporines (e.g., Sandimmune, cyclosporine analog, OG-37325, cyclosporin-G, and NVal-CyA), cytokines (e.g., IL-4 **fusion** toxin), cytokine receptor antagonists (e.g., immunomodulatory cytokines), E-selectin antagonists (e.g., anti-ELAM, and CY-1787), FK506/tacrolimus (e.g., Prograf), hypercalcemic agents (e.g., KH-1060), . . . converting enzyme inhibitors (ICE), IL-2 produced by E. coli (e.g., celmoleukin, IL-2, TGP-3, and Celeuk), immunoglobulins (e.g., anti-ELAM, CY-1788, and **humanized** CY-1787), immunostimulants (e.g., thymotrinan, RGH-0205, and TP3), immunosuppressants (e.g., Rapamycin, AY-22989, NSC-226080, NSC-606698, anti-CD4, T-cell inhibitor, anti-tac MAb, smart anti-tac MAb, Migis (membrane immunoglobulin-isotope specific) **antibodies**, SM-8849, immunophilins, VX-10367, VX-10393, VX-10428, mycophenolate mofetil, ME-MPA, RS-61444, cyclosporine, OL-27-400, Sandimmune, IL-4 **fusion** toxin, trypanosomal inhibitory factor (TIF), T-cell receptor, CD3/Ti, Org-4094, anti-TBM, CP 17193, Leflunomide/A-77-1726, ELAM-1, AnergiX, Spanidin, 15-deoxyspergualin, deoxyspurgiline, gusperimus hydrochloride, . . . immunotoxins (e.g., Zolimomab aritox, xmmly-h65-rta, xomazyme-lym/CD5-Plus, OrthoZyme-CD5+, XomaZyme-H65-rta, Xomazyme-CD5 Plus), intravenous immunoglobulins (e.g., IVIG), integrin antagonists (e.g., integrin blockers), Migis.TM. **antibodies**, monoclonal **antibody** therapeutics, murine MAb (e.g., anti-SLE vaccine, and MAb 3E10), primatized anti-CD4 **antibodies** (e.g., CE9.1), protease inhibitors (e.g., matrix metalloprotease (MMP) inhibitors, and stromelysin), protein synthesis antagonists (e.g., anti-CD6-bR, anti-T12-bR, and oncolysin CD6), . . . Cylexin), spergualin analogues (e.g., Spanidin, 15-deoxyspergualin, deoxyspurgiline, gusperimus hydrochloride, NSC-356894, and NKT-01), T cell inhibitors (e.g., AnergiX), tumor necrosis factor (**TNF**) antagonists, and the like;

L5 ANSWER 4 OF 7 MEDLINE
 ACCESSION NUMBER: 199268703 MEDLINE
 DOCUMENT NUMBER: 99268703

TITLE: Antitumor necrosis factor therapy for inflammatory bowel disease: a review of agents, pharmacology, clinical results, and safety [see comments].
COMMENT: Comment in: Inflamm Bowel Dis 2000 Feb;6(1):70
AUTHOR: Sandborn W J; Hanauer S B
CORPORATE SOURCE: Inflammatory Bowel Disease Clinic, Mayo Clinic and Mayo Foundation, Rochester, Minnesota 55905, USA.
SOURCE: INFLAMMATORY BOWEL DISEASES, (1999 May) 5 (2) 119-33.
Ref:

81
Journal code: C2I. ISSN: 1078-0998.
PUB. COUNTRY: United States
Journal; Article; (JOURNAL ARTICLE)
General Review; (REVIEW)
(REVIEW, TUTORIAL)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199909

AB . . . proinflammatory cytokine, plays an important role in the pathogenesis of inflammatory bowel disease (IBD). Biotechnology agents including a chimeric monoclonal anti-TNF antibody (infliximab), a humanized monoclonal anti-TNF antibody (CDP571), and a recombinant TNF receptor fusion protein (etanercept) have been used to inhibit TNFalpha activity. Controlled trials have demonstrated efficacy for infliximab in moderately to severely. . . RA patients who have failed disease modifying antirheumatic drug (DMARD) therapy leading to FDA approval for RA. Toxicities observed with anti-TNF therapies have included formation of human antichimeric antibodies (HACA) with associated acute and delayed hypersensitivity infusion reactions, human antihuman antibodies (HAHAs), and formation of autoantibodies with rare instances of drug-induced lupus. Several cases of non-Hodgkin's lymphoma also has been described. Future studies should evaluate optimal timing and duration of anti-TNF therapy, the utility of adjuvant medical treatments during anti-TNF therapy, and evaluate long-term safety and efficacy of the various anti-TNF agents.

L5 ANSWER 5 OF 7 CAPLUS COPYRIGHT 2000 ACS

ACCESSION NUMBER: 1999:414930 CAPLUS
DOCUMENT NUMBER: 131:241552
TITLE: New therapeutic targets for rheumatoid arthritis
AUTHOR(S): Dinant, H. J.; Dijkmans, B. A. C.
CORPORATE SOURCE: Department of Rheumatology, Jan van Breemen Institute,
Amsterdam, 1056 AB, Neth.
SOURCE: Pharm. World Sci. (1999), 21(2), 49-59
CODEN: PWSCED; ISSN: 0928-1231
PUBLISHER: Kluwer Academic Publishers
DOCUMENT TYPE: Journal; General Review
LANGUAGE: English
REFERENCE COUNT: 109
REFERENCE(S): (1) Allen, J; J Immunol 1993, V151, P4344 CAPLUS
(4) Bandara, G; DNA Cell Biol 1992, V11, P227 CAPLUS
(6) Bathon, J; Arthritis Rheum 1994, V37, P1350
CAPLUS
(7) Boers, M; Lancet 1997, V350, P309 CAPLUS
(8) Brennan, F; Scand J Immunol 1995, V42, P158
CAPLUS

ALL CITATIONS AVAILABLE IN THE RE FORMAT

AB A review with 109 refs. New insights into the pathogenesis of rheumatoid arthritis (RA) and consequently new targets of therapy are covered in a broad overview fashion. Short-term significant beneficial effect on RA disease activity has been established in a small but rapidly growing no. of double-blind placebo-controlled trials now including recombinant human IL-1 receptor antagonist, chimeric (mouse/human) monoclonal antibodies (mAb) against TNF.alpha. (cA2),

humanized (human/mouse) anti-**TNF**.alpha. mAb (CDP571) and recombinant human **TNF**-receptor-Fc **fusion** protein (TNFR: Fc). Placebo-controlled trials of anti-T cells agents such as chimeric anti-CD4 mAb (cM-T412) and anti-CD5 immunoconjugate, did not demonstrate clin. benefit. A placebo-controlled study of the anti-T cell derived cytokine IL-2 (DAB486IL-2) showed only modest clin. improvement. Other anti-T cell approaches such as autologous T cell vaccination and induction of tolerance by oral type II collagen have been unsuccessful. The one controlled trial with an anti-inflammatory cytokine, recombinant human IFN-.gamma., showed modest clin. benefits. Controlled trials with IL-4 and IL-10 and with anti-adhesion mols. are awaited.

L5 ANSWER 6 OF 7 MEDLINE DUPLICATE 1
 ACCESSION NUMBER: 93342357 MEDLINE
 DOCUMENT NUMBER: 93342357
 TITLE: [Anti-cytokines and anti-cytokine receptors].
 Anticytokines et antirecepteurs de cytokine.
 AUTHOR: Dantal J; Giral M; Souillou J P
 CORPORATE SOURCE: INSERM U211 Unite de recherche sur les effecteurs
 lymphocytaires T, Plateau technique, CHU Hotel-Dieu,
 Nantes..
 SOURCE: REVUE DU PRATICIEN, (1993 Mar 1) 43 (5) 586-9. Ref: 18
 Journal code: T1D. ISSN: 0035-2640.
 PUB. COUNTRY: France
 Journal; Article; (JOURNAL ARTICLE)
 General Review; (REVIEW)
 (REVIEW, TUTORIAL)
 LANGUAGE: French
 FILE SEGMENT: Foreign
 ENTRY MONTH: 199311
 AB Cytokines play a key-role in the immune response. The best known of them
 is interleukin-2 and its specific receptors. Monoclonal **antibodies**
 directed against the interleukin-2 receptor have initially enabled this
 receptor to be characterized; then they served to confirm the major. .

in man, particularly in kidney transplantation (but also in bone marrow
 transplantation), and they encourage to develop new bioreagents (chimeral
antibodies, "**humanized**" **antibodies**,
fusion proteins). Some of these reagents are now undergoing
 evaluation in renal transplantation. The principles of these bioreagents,
 issued from molecular. . . myeloma. Data from immune intervention
 directed against other cytokines are, for the moment, preliminary, but
 many potential targets (IL-1, IL-4, **TNF** alpha, INF gamma) are
 emerging.

L5 ANSWER 7 OF 7 MEDLINE DUPLICATE 2
 ACCESSION NUMBER: 91291906 MEDLINE
 DOCUMENT NUMBER: 91291906
 TITLE: Targeting of tumor necrosis factor to tumor cells:
 secretion by myeloma cells of a genetically engineered
 antibody-tumor necrosis factor hybrid molecule.
 AUTHOR: Hoogenboom H R; Raus J C; Volckaert G
 CORPORATE SOURCE: Dr. L. Willems-Instituut en Departement WNIF, Limburgs
 Universitair Centrum, Diepenbeek, Belgium.
 SOURCE: BIOCHIMICA ET BIOPHYSICA ACTA, (1991 Jun 5) 1096 (4)
 345-54.
 Journal code: A0W. ISSN: 0006-3002.
 PUB. COUNTRY: Netherlands
 Journal; Article; (JOURNAL ARTICLE)
 LANGUAGE: English
 FILE SEGMENT: Priority Journals; Cancer Journals
 ENTRY MONTH: 199110
 AB The construction, synthesis and secretion of a genetically engineered
antibody-cytokine **fusion** molecule is described. To
 target tumor necrosis factor (**TNF**) to tumor cells, recombinant
antibody techniques were used to produce a Fab-like

antibody-TNF conjugate. At the gene level, the heavy chain gene of an antitransferrin receptor **antibody** was linked to a synthetic **TNF** gene encoding human **TNF**. Transfection of the heavy chain-**TNF** gene into a myeloma derived cell line which was producing the light chain of the same **antibody**, allowed the isolation of a cell line secreting a **fusion** protein of the expected molecular weight and composition. The culture supernatant of the cell line contained **TNF** cytotoxic activity towards murine L929 cells and human MCF-7 cells. Cytotoxicity towards the human cancer cells was inhibited by an excess of the original antitransferrin receptor **antibody**, indicating that the **antibody-TNF** molecules are targeted to the transferrin receptor rich tumor cells.

Since

the **antibody** genes used are chimeric (i.e. composed of mouse variable and human constant regions) and since DNA encoding human **TNF** was used, the hybrid protein is an example of a **humanized** immunotoxin-like molecule. These results illustrate the possibilities of **antibody** engineering technology to create and produce improved agents for cancer therapy. Furthermore, they demonstrate for the first time the ability of myeloma cells to secrete an **antibody**-cytokine chimeric molecule.

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(FILE 'HOME' ENTERED AT 16:05:52 ON 16 AUG 2000)

FILE 'MEDLINE, BIOSIS, EMBASE, CAPLUS, USPATFULL' ENTERED AT 16:06:12 ON 16 AUG 2000

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L1      18 S ANTIBODY (P) HUMANIZED (P) FUSION (P) INTERLEUKIN
L2      10 DUP REM L1 (8 DUPLICATES REMOVED)
L3      90 S ANTIBODY (P) HUMANIZED (P) TNF
L4      11 S ANTIBODY (P) HUMANIZED (P) TNF (P) FUSION
L5       7 DUP REM L4 (4 DUPLICATES REMOVED)
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=> s antibody (p) humanized (p) lymphokine (p) fusion

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L6      3 ANTIBODY (P) HUMANIZED (P) LYMPHOKINE (P) FUSION
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=> dup rem l6

PROCESSING COMPLETED FOR L6

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L7      3 DUP REM L6 (0 DUPLICATES REMOVED)
```

=> d l7 total ibib kwic

L7 ANSWER 1 OF 3 USPATFULL

ACCESSION NUMBER: 2000:41155 USPATFULL

TITLE: FAS ligand fusion proteins and their uses

INVENTOR(S): Queen, Cary L., Los Altos, CA, United States

Schneider, William P., Los Altos, CA, United States

Vasquez, Maximiliano, Palo Alto, CA, United States

PATENT ASSIGNEE(S): Protein Design Labs., Inc., Fremont, CA, United States
(U.S. corporation)

	NUMBER	DATE
PATENT INFORMATION:	US 6046310	20000404
APPLICATION INFO.:	US 1997-815190	19970311 (8)
RELATED APPLN. INFO.:	Continuation-in-part of Ser. No. US 1996-614584, filed on 13 Mar 1996, now abandoned	
DOCUMENT TYPE:	Utility	
PRIMARY EXAMINER:	Chan, Christina Y.	
ASSISTANT EXAMINER:	Nolan, Patrick J.	

LEGAL REPRESENTATIVE: Townsend & Townsend & Crew LLP
NUMBER OF CLAIMS: 8
EXEMPLARY CLAIM: 1
NUMBER OF DRAWINGS: 6 Drawing Figure(s); 7 Drawing Page(s)
LINE COUNT: 1454

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

DETD In one aspect, the present invention is directed to a **fusion** protein comprising a functional moiety of the extracellular domain of the FasL protein and a polypeptide capable of specifically binding. . . antigen, expressed on particular cell-type(s) or tissue(s). In a preferred embodiment, the binding polypeptide comprises the variable domain of an **antibody**. In a particularly preferred embodiment, the **antibody** is **humanized**, human or from another primate species. However, the binding polypeptide may also comprise, for example, the binding site of a cellular receptor; a receptor ligand such as a cytokine, **lymphokine**, interleukin, growth factor, hormone or the like; or the binding site of an adhesion molecule, such as a selectin or. . . region (e.g., an integral membrane protein or a transmembrane glycoprotein), wherein said extracellular portion can be specifically bound by an **antibody** or other ligand, i.e., with an affinity of stronger than about 1.times.10.sup.6 M.sup.-1. The term cell surface marker also refers. . . specificity of an intact FasL polypeptide, but will be soluble rather than membrane bound. Preferably, the FasL component of the **fusion** protein is found within a segment of up to about 10, 25 or 50 amino acids within the FasL extracellular. . .

L7 ANSWER 2 OF 3 USPATFULL

ACCESSION NUMBER: 1999:27414 USPATFULL
TITLE: Monoclonal antibodies specific for different epitopes of human GP39 and methods for their use in diagnosis and therapy
INVENTOR(S): Siadak, Anthony W., Seattle, WA, United States
Hollenbaugh, Diane L., Seattle, WA, United States
Gilliland, Lisa K., Bellevue, WA, United States
Gordon, Marcia L., Seattle, WA, United States
Bajorath, Jurgen, Lynnwood, WA, United States
Aruffo, Alejandro A., Edmonds, WA, United States
PATENT ASSIGNEE(S): Bristol-Myers Squibb Company, New York, NY, United States (U.S. corporation)

	NUMBER	DATE
PATENT INFORMATION:	US 5876950	19990302
APPLICATION INFO.:	US 1995-379057	19950126 (8)
DOCUMENT TYPE:	Utility	
PRIMARY EXAMINER:	Saunders, David	
LEGAL REPRESENTATIVE:	Klein, Christopher A.; Poor, Brian W.; Sorrentino, Joseph M.	
NUMBER OF CLAIMS:	50	
EXEMPLARY CLAIM:	1	
NUMBER OF DRAWINGS:	16 Drawing Figure(s); 14 Drawing Page(s)	
LINE COUNT:	3341	

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

DETD The monoclonal **antibodies**, particularly the recombinant binding proteins, single chain **antibodies**, chimeric **antibodies** and **humanized antibodies**, of this invention can also be incorporated as components of pharmaceutical compositions containing an amount of binding protein which is. . . adjuvants (buffering agents, dispensing agents) may also be incorporated into the pharmaceutical composition. Such compositions can contain a single monoclonal **antibody** or recombinant binding protein

specific for human gp39. Alternatively, a pharmaceutical composition
can contain other biologically active molecules, for example,
lymphokines, cytokines, other monoclonal **antibodies** or
fusion proteins (i.e., CD28-Ig, CTLA4-Ig).

L7 ANSWER 3 OF 3 CAPLUS COPYRIGHT 2000 ACS

ACCESSION NUMBER: 1991:447551 CAPLUS

DOCUMENT NUMBER: 115:47551

TITLE: Targeting of tumor necrosis factor to tumor cells:
secretion by myeloma cells of a genetically

engineered

antibody-tumor necrosis factor hybrid molecule
AUTHOR(S): Hoogenboom, Hennie R.; Raus, Jef C. M.; Volckaert,
Guido

CORPORATE SOURCE: Dr. L. Willems-Inst., Limburgs Univ. Cent.,
Diepenbeek, B-3610, Belg.

SOURCE: Biochim. Biophys. Acta (1991), 1096(4), 345-54
CODEN: BBACAQ; ISSN: 0006-3002

DOCUMENT TYPE: Journal

LANGUAGE: English

IT **Lymphokines** and Cytokines

RL: BIOL (Biological study)

(tumor necrosis factor, **fusion** product, with

humanized monoclonal **antibody** Fab fragment to

transferrin receptor, genetic engineering of and human breast
adenocarcinoma inhibition by)

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COST IN U.S. DOLLARS

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FULL ESTIMATED COST

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DISCOUNT AMOUNTS (FOR QUALIFYING ACCOUNTS)

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SESSION

CA SUBSCRIBER PRICE

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STN INTERNATIONAL LOGOFF AT 16:10:55 ON 16 AUG 2000 .

STIC-ILL

308107

From: Murphy, Joseph
Sent: Wednesday, August 16, 2000 4:30 PM
To: STIC-ILL
Subject: 09256156

Please send me the following references:

TITLE: Preparation and characterization of a recombinant humanized single-chain Fv antibody/human interleukin -2 fusion protein directed against the HER-2/neu (c-erbB2) proto-oncogene product, p185.
AUTHOR: Li J.; Gyorffy S.F.; Ring D.B.; Kwok C.S.; Austin R.C.
SOURCE: Tumor Targeting, (1999) 4/2 (105-114).

TITLE: Humanized monoclonals and other biological initiatives.
AUTHOR(S): Halloran, Philip F. (1); Prommool, Surazee
SOURCE: Clinical Biochemistry, (July, 1998) Vol. 31, No. 5, pp. 353-357.

TITLE: Lymphokine receptor-directed therapy: a model of immune intervention.
AUTHOR: Waldmann T A; Grant A; Tendler C; Greenberg S; Goldman C; Bamford R; Junghans R P; Nelson D
SOURCE: JOURNAL OF CLINICAL IMMUNOLOGY, (1990 Nov) 10 (6 Suppl) 19S-28S

Thanks a lot...

Joseph F. Murphy, Ph.D.
Patent Examiner, Art Unit 1646
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(703) 305-7245

Bu

ND 8/17



PAPER

Preparation and characterization of a recombinant humanized single-chain Fv antibody/human interleukin-2 fusion protein directed against the *HER-2/neu* (*c-erbB2*) proto-oncogene product, p185¹

J Li¹, SF Gyorffy¹, DB Ring², CS Kwok³ and RC Austin^{1*}

¹Hamilton Civic Hospitals Research Centre and Department of Pathology, McMaster University, Ontario, Canada;

²Department of Immunology, Chiron Corporation, Emeryville, CA, USA; and ³Department of Optometry and Radiography, Hong Kong Polytechnic University, Kowloon, Hong Kong

High dose recombinant human interleukin 2 (rhIL-2) therapy has been used in the treatment of established tumors in both animal models and patients with advanced melanoma or renal carcinoma. However, because high dose rhIL-2 therapy causes severe systemic toxicity in normal tissues, its clinical use has been limited. Therefore, targeting interleukin-2 (IL-2) to the tumor site should improve its anti-tumor-immune response and decrease its systemic toxicity. In this study, we describe the preparation and characterization of a recombinant humanized single-chain Fv (sFv) antibody/IL-2 fusion protein. This recombinant fusion protein consists of humanized variable heavy (V_H) and light (V_L) domains of monoclonal antibody (mAb) 520C9 directed against the human *HER-2/neu* (*c-erbB2*) proto-oncogene product p185 and human IL-2. The fusion protein was stably expressed in baby hamster kidney cells and shown to retain the immunostimulatory activities of IL-2 as measured by IL-2-dependent cell proliferation and cytotoxicity assays. In addition to its IL-2 activity, this fusion protein also possesses binding specificity against the *HER-2/neu* (*c-erbB2*) proto-oncogene product, p185, as determined by enzyme linked immunosorbent assay (ELISA) using SKOV 3ip1 cells. Taken together, these findings suggest that this recombinant humanized sFv antibody/IL-2 fusion protein may provide an effective means of targeting therapeutic doses of IL-2 to p185 positive tumors without increasing systemic toxicity or immunogenicity.

Keywords: interleukin-2; humanized sFv; fusion protein; *HER-2/neu* (*c-erbB2*) proto-oncogene

*Correspondence: RC Austin, Hamilton Civic Hospitals Research Centre, 711 Concession St., Hamilton, Ontario, Canada L8V 1C3.

Introduction

Interleukin-2 (IL-2) is a 15 kDa cytokine synthesized and secreted primarily by activated T cells.^{1,2} IL-2 occupies a central role in the augmentation of cell mediated immune responses by stimulating and supporting a broad range of immune cells *in vitro* and *in vivo*,³ including T and B cells, macrophages, and natural killer (NK) cells. Furthermore, IL-2 can stimulate the production of lymphokine activated killer (LAK) cells, which are derived from NK cells and can effectively mediate antibody-dependent cellular toxicity.

The administration of recombinant human IL-2 (rhIL-2), in combination with LAK cells, has been shown to be of value in the treatment of patients with advanced melanoma or renal cell carcinoma.⁴ However, the *in vivo* efficacy of rhIL-2 treatment has been limited by its associated severe systemic toxicity and difficulties in maintaining prolonged high concentration of the cytokine in the tumor microenvironment, which is likely necessary to induce local anti-tumor immune responses.^{5,6} To circumvent these problems, several approaches to selectively target IL-2 to tumor sites have been employed, particularly the use of immunoconjugates.⁷⁻⁹

Murine monoclonal antibodies (mAbs) have been extensively used as carriers to target therapeutic agents to tumor sites for diagnostic and therapeutic modalities over the past decade. Despite some highly encouraging diagnostic data obtained with this approach, the general therapeutic efficacy has been rather disappointing.¹⁰⁻¹² Several major obstacles related to the mAb approach have been identified, including relatively long half-life of the immunocomplex, human anti-mouse antibody (HAMA) response and inability of the immunoconjugate to penetrate large tumor masses.^{13,14} To date, several approaches of engineering conventional murine mAbs have been developed for more effective cancer targeting therapy. One approach is the development of single-chain Fv (sFv) versions of the intact antibody consisting of the V_H and V_L domains of the antibody linked by a flexible peptide spacer. This version of antibody is the smallest antibody fragment capable of binding antigen. Advantages of this small antibody fragment include improved clearance of immunocomplex from the circulation, better penetration in solid tumors and lower

immunogenicity.¹⁵⁻¹⁷ Other approaches include the development of recombinant immunotoxins¹⁸ and antibody immunostimulatory molecule conjugates.^{19,20}

In this report, we describe the preparation and characterization of a recombinant humanized sFv antibody/hIL-2 fusion protein by combining the humanized V_H and V_L domains of 520C9, a murine mAb (IgG₁) directed against the human *HER 2/neu (c-erbB2)* proto-oncogene product, p185,²¹⁻²⁵ to human IL-2. This fusion protein was shown to have IL-2 bioactivity characterized by the proliferation of IL-2-dependent cytotoxic T cell lines (CTLL) cells and cytotoxicity assays,^{2,26,27} as well as antigen-binding activity determined by indirect ELISA using p185 positive SKOV 3ip1 cells. These findings suggest that this humanized sFv antibody/hIL-2 fusion protein may provide an effective method of targeting therapeutic doses of rIL-2 to p185 positive tumors while significantly reducing its systemic toxicity and immunogenicity.

Materials and methods

Construction of expression plasmids

Mammalian expression plasmids encoding the humanized sFv antibody/IL-2 fusion proteins were constructed as described in Figure 1. Baculovirus expression plasmid pAcHCs-520C9sFv containing a humanized 816-bp 520C9 sFv cDNA fragment, and the plasmids pLW46 and plasmid pLW42 containing either wild type or mutant human IL-2 cDNA fragments, respectively, were kindly provided by Chiron Corporation (Emeryville, CA). The mutant-human IL-2 cDNA contains a single base substitution at position 173 (G→C) which converts amino acid Cys 58 of the wild type protein to Ser, therefore eliminating a disulphide bond critical to the biological function of IL-2. Primers used for the amplification of 520C9 sFv and human IL-2 cDNA fragments from the above plasmids were synthesized at the Institute for Molecular Biology (MOBIX), McMaster University (Hamilton, ON). The forward primer AB9883 (5'-CTT AAG CTT GCC ACC ATG GAC ATG AGG GTC CCC GCT-3') used to amplify the sFv cDNA contained a Kozak consensus sequence prior to the initiating ATG and a terminal *Hind* III restriction site. The reverse primer AB7824 (5'-CC GAA TTC TTT AAT CTC CAG TTT TGT CCC TTG GGC-3') contained an *Eco*RI site 3' to the sFv cDNA fragment. The 440-bp mutant human IL-2

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and wild type human IL-2 cDNA fragments derived from pLW42 and pLW46 were subjected to polymerase chain reaction (PCR) amplification using the forward primer AB7822 (5'-GGG GAA TTC GGT GGC GGT GGC TCG GGC GGT GGT GGC TCG GGT GGC GGC GGA TCT ATG CCT ACT TCA AGT TCT ACA AAG AAA ACA CAG-3'), which contained a terminal *EcoRI* site and a coding sequence for a 15 amino acid, glycine rich linker peptide (NH₂-GGGSGGGGSGGGGS-COOH)5' to the human IL-2 cDNA

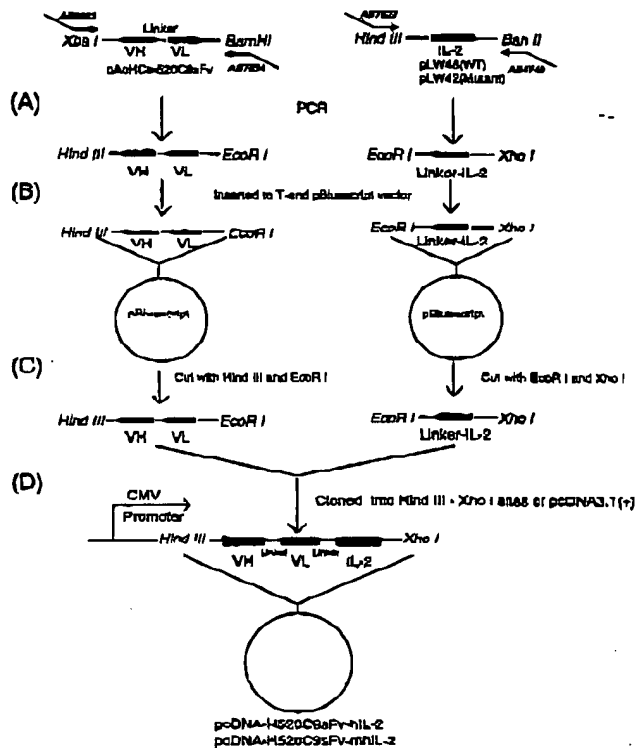


Figure 1 Schematic diagram of the construction of expression plasmids.

A: primers AB9883 and AB7824 were used to amplify and modify humanized mAb 520C9 sFv cDNA from the plasmid pAcHCs-520C9sFv. Primers AB7822 and AB4749 were used for amplification and modification of wild type (WT) or mutant human IL-2 cDNA fragments from the plasmids pLW46 and pLW42, respectively. B: PCR-amplified cDNA fragments were inserted into T-ended pBluescript. C: pBluescript containing the cDNA inserts were digested with *HindIII*-*EcoRI* or *EcoRI*-*XhoI* and purified from agarose gels. D: cDNA fragments were then ligated into the *HindIII*-*XhoI* sites of the mammalian expression vector pcDNA3.1(+) to generate the expression plasmids pcDNA-H520C9sFv-hIL-2 or pcDNA-H520C9sFv-mhIL-2.

fragment. The reverse primer AB4749 (5'-GAC CTC GAG TCA GTG TTG AGA TGA T-3') contained a terminal *XhoI* site adjacent to the termination codon of the human IL-2 cDNA fragment. PCR products were purified from 0.8% agarose gels and ligated into T-ended pBluescript using T4 DNA ligase (Gibco/BRL, Burlington, ON). The ligation mixtures were then used to transform competent DH5 α cells (Gibco/BRL). Plasmids with inserts were digested with *HindIII* and *EcoRI* for the sFv, or *EcoRI* and *XhoI* for the human IL-2 cDNA fragments. These cDNA fragments were purified from agarose gels using QIAGEN Gel Purification Kit (Qiagen, Mississauga, ON) and ligated into the *HindIII*/*XhoI* site of the mammalian expression vector pcDNA3.1(+) (Invitrogen, Carlsbad, CA) to produce the recombinant plasmids, humanized 520C9 sFv/human interleukin-2 fusion protein (pcDNA-H520C9sFv-hIL-2) and humanized 520C9 sFv/mutant human interleukin-2 fusion protein (pcDNA-H520C9sFv-mhIL-2). Fidelity of the PCR-amplified constructs was confirmed by fluorescence-based double-stranded DNA sequencing (MOBIX). The ligation mixture was then used to transform competent DH5 α cells. Transformants containing both the humanized 520C9 sFv and IL-2 cDNA fragments were confirmed by restriction enzyme analysis. The constructs were subsequently purified using QIAGEN Plasmid Midi Kits and dissolved in Tris-EDTA buffer (pH 7.4) to a concentration of 0.4 mg/ml.

Establishment of stable cell lines

Baby hamster kidney (BHK) cells used for stable expression of the cDNAs encoding H520C9sFv-hIL-2 or H520C9sFv-mhIL-2 were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco/BRL) containing 10% fetal calf serum (FCS; HyClone Laboratories, Logan, UT) at 37°C in a 5% CO₂ incubator. Cells grown to 20% confluency were transfected with 5 μ g of the plasmid constructs using 30 μ l of SuperFect Transfection Reagent (Qiagen) as described by the manufacturer. As a negative control, pcDNA3.1(+) was used to transfect BHK cells under the same conditions. Stable transfectants were selected in DMEM containing 10% FCS and 600 μ g/ml G418 (Gibco/BRL) for two weeks, after which the concentration of G418 was increased to 1.2 mg/ml. Clones secreting either the H520C9sFv-hIL-2 or the



H520C9sFv-mhIL-2 fusion protein were identified by ELISA using either a mouse anti-human IL-2 mAb MAB202 (R&D Systems, Minneapolis, MN) or a rabbit anti-human IL-2 polyclonal antibody EP100 (Genzyme, Cambridge, MA). In brief, 100 µl of supernatant from different clones was added to a 96-well plate coated with MAB202 (1:250 dilution), and incubated at 37°C for 2 h. After three washes with phosphate-buffered saline containing 0.05% Tween-20 (PBST), the plate was incubated with EP100 (1:200 dilution) at 37°C for 1 h, washed and then incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG polyclonal antibody (Gibco/BRL) (1:1000 dilution) for 1 h at 37°C. After three additional washes with PBST, colour development was performed with peroxidase substrate (Bio-Rad, Hercules, CA) and the resulting optical density (OD) determined at 405 nm in a microplate reader (Bio-Tek Instrument Inc., Winooski, VT). Clones producing the highest amount of fusion protein were selected and grown in DMEM containing 10% FCS for 72 h. The conditioned medium was collected for subsequent precipitation and analysis.

Immunoprecipitation and purification of fusion proteins

Fusion proteins were immunoprecipitated using MAB202 coupled with CNBr-activated Sepharose CL-4B (Pharmacia Biotech, Baie d'Urfé, QC). 25 µl of a 1:1 (v/v) slurry of the absorbent suspended in 20 mM Tris-HCl buffer (pH 7.8) was added to 1.5 ml of the conditioned medium with gentle shaking at 4°C for 4 h. The Sepharose beads were washed three times using Tris-HCl buffer, then 50 µl of 1X SDS-PAGE sample buffer containing β-mercaptoethanol was added to the beads. After heating for 3 min at 100°C, the beads were pelleted by centrifugation (12 000 × g, 2 min) and the supernatant collected for immunoblot analysis.

For affinity purification of the fusion protein, the conditioned medium was applied to a MAB202-Sepharose CL-4B affinity column at a flow rate of 5 column vol/h. The column was then washed three times with 20 mM Tris-HCl buffer (pH 7.8) and the fusion protein was eluted with Pierce Gentle Ag/Ab Elution Buffer (Rockford, IL) as described by the manufacturer.

Immunoblot analysis

20 µl of 50-fold concentrated conditioned media or 20 µl of the immunoprecipitate were heated to 100°C for 3 min and then separated on a 15% SDS-polyacrylamide gel under reducing conditions. Proteins were electroblotted onto nitrocellulose membranes (Bio-Rad, Richmond, CA) and the efficiency of protein transfer was determined by staining the blots with Ponceau S (Sigma, St. Louis, MO). Membranes were washed briefly in Tris-buffered saline containing 0.05% Tween-20 (TBST) to remove the Ponceau S stain, blocked in TBST containing 5% skimmed milk at 4°C overnight and incubated at room temperature for 2 h with EP100 (1:1000 dilution). Following three 20 min washes in TBST, the membranes were incubated for 2 h with a HRP-conjugated goat anti-rabbit IgG polyclonal antibody (1:2000 dilution). After washing in TBST, membranes were developed with the Renaissance Chemiluminescence Reagent Kit (DuPont, Boston, MA) as described by the manufacturer.

IL-2 bioactivity assay

Biological activity of the fusion proteins was determined by standard IL-2-dependent CTLL-2 cell proliferation² and cytotoxicity assays.²³ CTLL-2 cells deprived of rhIL-2 (Chiron, Emeryville, CA) for 3 d were seeded into a 96-well plate at 1×10^4 cells per well and incubated with either serially diluted rhIL-2 standard or fusion protein at 37°C in a 5% CO₂ incubator. After 24 h, 1 µCi of [³H]-thymidine (DuPont, Boston, MA) was added to each well and the cells incubated for an additional 12 h. Cells were harvested onto glass fibre filters (Whatman Corp., Walkersville, MD), dried and the incorporated radioactivity counted in a Beckman scintillation counter (Fullerton, CA).

For bioactivity neutralization experiments, 2 ng/ml of rhIL-2 and the same concentration of IL-2 in H520C9sFv-hIL-2 were incubated with increasing concentrations of the anti-rhIL-2 neutralizing mAb MAB202 for 2 h at 37°C in a 96-well microtiter plate. As a control, mouse IgG was added to H520C9sFv-hIL-2 instead of MAB202. Following this incubation period, 1×10^4 CTLL-2 cells were added to each well. The assay mixture, in a total volume of 100 µl/well, was incubated for 24 h at 37°C in a 5% CO₂ incubator and pulsed with

1 μCi /well [^3H]-thymidine for the final 12 h. The cells were then harvested onto glass fiber filters and [^3H]-thymidine incorporation was determined.

In the cytotoxicity assay, peripheral blood mononuclear cells (PBMCs) from healthy individuals were separated on a Histopaque 1077 density gradient (Sigma, St. Louis, MO). The PBMCs were cultured in RPMI 1640 medium (Gibco/BRL) supplemented with 10% FCS containing either 1000 IU/ml rhIL-2 or same dose of the H520sFv-hIL-2 fusion protein as determined by the above CTL-2 assay for 3 d to produce LAK cells. Cytotoxicity of the resulting LAK cells was assessed by a 4 h Calcein AM (Molecular Probes, Eugene, OR) release assay with NK-resistant Daudi cells (ATCC, Rockville, MD) used as target cells. Each well of a 96-well plate contained 1×10^4 Daudi cells which had been pre-labelled with fluorescent Calcein AM. To achieve different effector/target cell ratios, an increasing number of LAK cells were added to each well. The total volume of culture medium per well was 0.1 ml. Following co-incubation of LAK and Daudi cells at 37°C for 4 h, the cells were pelleted and the fluorescent intensity value ($\text{Value}_{\text{sample}}$) of 50 μl of cell supernatant from each well containing both types of cells was measured with a Cytofluor 2300 fluorescence reader system (Millipore, Bedford, MA). The maximal release value ($\text{Value}_{\text{maximal}}$) of the fluorescent tag was determined by adding an equal volume of 1% (v/v) Triton X-100 to the wells with only target cells. The spontaneous release value ($\text{Value}_{\text{spontaneous}}$) of the tag was given by the fluorescent intensity of 50 μl of cell supernatant from the wells without any LAK cells. The percent specific lysis for each sample of LAK cells was calculated by the formula $(\text{Value}_{\text{sample}} - \text{Value}_{\text{spontaneous}})/(\text{Value}_{\text{maximal}} - \text{Value}_{\text{spontaneous}}) \times 100$.

Scatchard analysis

Scatchard binding assays for the original 520C9 mAb, the humanized 520C9 sFv fragment and the proteolytically cleaved Fab fragment of 520C9 were performed on SK-Br-3 cells as described previously.²⁸

Determination and specificity of antigen-binding activity of H520C9sFv-hIL-2

Antigen-binding activity was measured by indirect ELISA using cultured SKOV3ip1 or HeLa cells.

Human ovarian carcinoma SKOV3ip1 cells which express high levels of p185,²⁹ and HeLa cells which express undetectable levels of p185, were maintained in DMEM containing 10% FCS and used for determination of antigen-binding activity of the H520C9sFv-hIL-2 fusion protein. Cells (1×10^4) were added to individual wells of a 96-well microtiter plate one day before the assay. Serially diluted samples of the H520C9sFv-hIL-2 or a chemically-conjugated control mAb 520C9-rhIL-2 (Li et al, unpublished data) were added to each well of the plate and incubated at 37°C for 2 h. After three washes with PBST, EP100 (1:250 dilution) was added to the cells and incubated for 2 h. Following three washes with PBST, HRP-conjugated goat anti-rabbit IgG polyclonal antibodies (Gibco/BRL) (1:1000 dilution) were added to the cells. After 2 h incubation, the cells were washed three times with PBST, colour was developed with the addition of peroxidase substrate (Bio-Rad, Hercules, CA) and the OD of the solution was determined at 405 nm in a microplate reader as described.

To demonstrate specificity for p185 binding, SKOV3ip1 cells (1×10^4) grown in 96-well plates were treated with either serially diluted intact 520C9 mAb or normal mouse IgG (0.001 to 10 nM) at 37°C for 2 h prior to the addition of 10 nM H520C9sFv-hIL-2 fusion protein. Indirect ELISA was then performed as described above.

Statistical analysis

Data are presented as means \pm standard deviation (s.d.). Statistical differences between the groups were determined by analysis of variance. If a significant difference between these groups was demonstrated, an unpaired Student's *t*-test was performed for each point. For all analyses, $P < 0.05$ were considered statistically significant.

Results

Detection of the IL-2 moiety in the fusion protein

To confirm the presence of the IL-2 moiety in the fusion protein, Western blot and immunoprecipitation analyses were performed using the anti-human IL-2 antibodies, EP100 (rabbit anti-human IL-2 Ab) and MAB202 (mouse anti-human IL-2 mAb). Conditioned media from BHK cells stably transfected with either pcDNA3.1(+), pcDNA-H520C9sFv-hIL-2 or pcDNA-

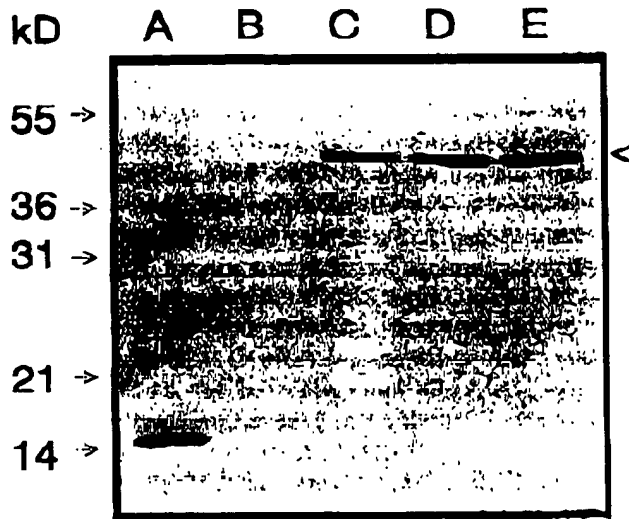


Figure 2 Western blot analysis of recombinant humanized sFv antibody/IL-2 fusion proteins stably expressed in BHK cells.

Lane A: 0.5 μ g of rhIL-2. Lanes B, C and D: conditioned medium from BHK cells transfected with either pcDNA3.1(+), pcDNA-H520C9sFv-hIL-2 or pcDNA-H520C9sFv-mhIL-2, respectively. Lane E: H520C9sFv-hIL-2 fusion protein immunoprecipitated with anti-human IL-2 mAb, MAB202. Samples were separated on a 15% SDS-polyacrylamide gel under reducing conditions, transferred to nitrocellulose membranes and immunoblotted using the anti-human IL-2 polyclonal antibody, EP100. The fusion proteins, H520C9sFv-hIL-2 and H520C9sFv-mhIL-2 were shown to migrate as single bands of 45 kD (arrowhead).

H520C9sFv-mhIL-2, and the MAB202 immunoprecipitate were analysed by Western blotting using EP100. As shown in Figure 2, a single band of 45 kD was observed in the conditioned medium from BHK cells transfected with either pcDNA-H520C9sFv-hIL-2 (lane C), pcDNA-H520C9sFv-mhIL-2 (lane D), or the MAB202 immunoprecipitate (lane E), but not in the conditioned medium from BHK cells transfected with pcDNA3.1(+) (lane B). Furthermore, the migration positions of these fusion proteins on SDS-polyacrylamide gels are consistent with their predicted molecular mass. As a positive control, EP100 also recognized rhIL-2 (lane A).

Cell proliferation and cytotoxicity assays

The concentration of IL-2, as a constitutive component of H520C9sFv-hIL-2 in the conditioned medium

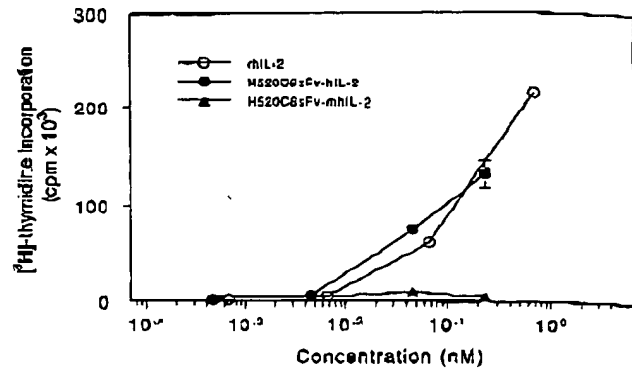


Figure 3 IL-2 activity of recombinant humanized sFv antibody/IL-2 fusion proteins.

IL-2 activity of rhIL-2 (○), or conditioned medium from BHK cells transfected with either pcDNA-H520C9sFv-hIL-2 (●) or pcDNA H520C9sFv-mhIL-2 (▲) was measured by [³H]-thymidine incorporation in CTLL-2 cells. The data are the mean \pm s.d. from three separate experiments.

was approximately 0.467 nM (data not shown) as determined by quantitative indirect ELISA. The activity of the H520C9sFv-hIL-2 fusion protein to support the proliferation of IL-2-dependent CTLL-2 cells and to generate LAK cells was compared to those of rhIL-2. As shown in Figure 3, conditioned medium from BHK cells transfected with pcDNA-H520C9sFv-hIL-2, but not pcDNA-H520C9sFv-mhIL-2, possessed similar IL-2 bioactivity to rhIL-2-standard. These results indicated that the bioactivity of the IL-2 moiety in terms of its ability to support the growth of CTLL-2 cells was maintained in the H520C9sFv-hIL-2 fusion protein.

In the cytotoxicity assay directed against target Daudi cells, LAK cells generated by 1000 IU/ml of IL-2 of the H520C9sFv-hIL-2 fusion protein, as determined by the above CTLL-2 assay, demonstrated similar effect in the killing of Daudi cells compared to LAK cells activated by 1000 IU/ml of rhIL-2 (Figure 4). The specific lysis of Daudi cells by the LAK cells stimulated by the H520C9sFv-hIL-2 and rhIL-2 were $76.3 \pm 1.7\%$ and $71.7 \pm 2.3\%$, respectively, at an effector:target ratio of 50:1. No cytotoxic effect was observed when conditioned medium from control BHK cells transfected with pcDNA3.1(+) was used.

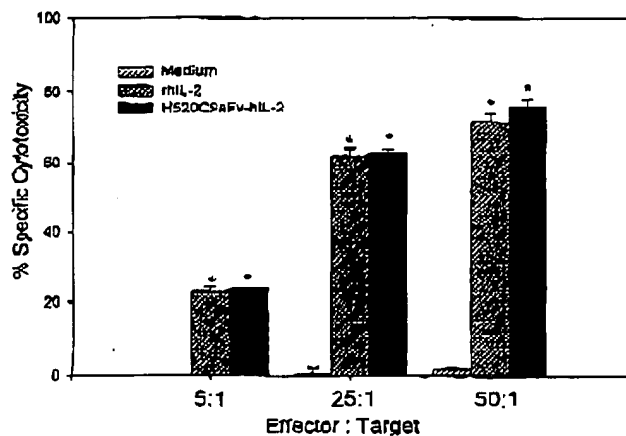


Figure 4 LAK cell-mediated cytotoxicity induced by H520C9sFv-hIL-2 fusion protein.

Human LAK cells (effector) were generated by incubation of fresh PBMCs with either conditioned medium from control BHK cells, rhIL-2 or equivalent dose of H520C9sFv-hIL-2. Lysis of Daudi cells (target) was determined using a 4 h Calcein AM-release assay. The data are the mean \pm s.d. from three separate experiments. * $P < 0.01$ vs medium from cells transfected with pcDNA 3.1 (+).

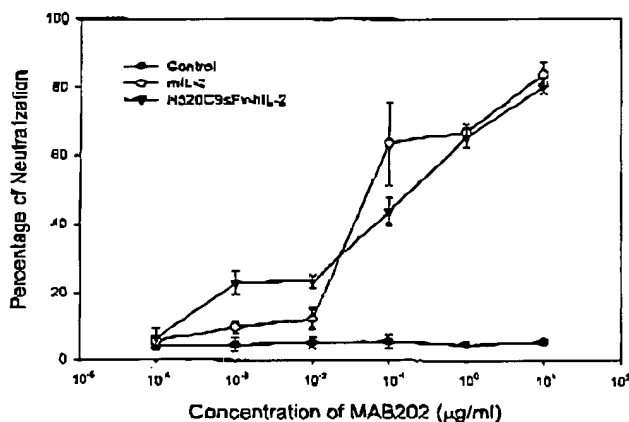


Figure 5 Inhibition of stimulatory effects of rhIL-2 (○) or H520C9sFv-hIL-2 (▼) on CTLL-2 cells by the anti-human IL-2 neutralizing antibody, MAB202.

Based on the molecular mass of either IL-2 or H520C9sFv-hIL-2 fusion protein, the Neutralization Dose 50 for 2 ng/ml rhIL-2 or the same dose of H520C9sFv-hIL-2 was determined to be approximately 0.076 and 0.128 μ g/ml MAB202, respectively. No inhibitory effect was observed using control mouse IgG (●). The data are the mean \pm s.d. from three separate experiments.



Neutralization of IL-2 activity in the H520C9sFv-hIL-2 fusion protein

To determine if the proliferation effects of the H520C9sFv-hIL-2 fusion protein were strictly IL-2 dependent, inhibition studies using IL-2 neutralizing antibodies were performed. As shown in Figure 5, the proliferative effects of both rhIL-2 and H520C9sFv-hIL-2 on CTLL-2 cells, as measured by [3 H]-thymidine incorporation of the CTLL-2 cells, were inhibited by MAB202. Approximately 50% inhibition of [3 H]-thymidine incorporation for 2 ng/ml rhIL-2 or same dose of H520C9sFv-hIL-2 was achieved at a MAB202 concentration of approximately 0.076 and 0.128 μ g/ml, respectively. No inhibitory effect was observed when control mouse IgG was added.

Scatchard analysis and antigen-binding specificity

Scatchard analysis using SK-Br-3 cells showed that the parental 520C9 mAb had an affinity constant (K_a) of 3.5×10^{-8} M. Although the K_a of the recombinant humanized 520C9 sFv protein (1.1×10^{-8} M) was slightly lower than that observed for the parental

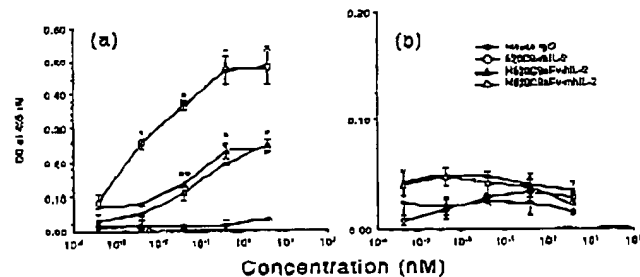


Figure 6 Determination of antigen-binding activity of H520C9sFv-hIL-2 fusion protein.

Antigen-binding activity was measured by indirect ELISA using either cultured SKOV 3ip1 or HeLa cells. Bound fusion protein was recognised with the anti-human IL-2 polyclonal antibody EP100. The fusion proteins, H520C9sFv-hIL-2 (▲) and H520C9sFv-mhIL-2 (Δ) were shown to bind to p185 positive cells SKOV 3ip1 (panel a), but not p185 negative HeLa cells (panel b). A chemically-conjugated molecule containing the intact parental 520C9 mAb and rhIL-2 (○) was used as a positive control. As a negative control, mouse IgG (●) failed to show any binding activity in both cell lines. The data are the mean \pm s.d. from three separate experiments. * $P < 0.01$ vs normal mouse IgG. ** $P < 0.05$ vs normal mouse IgG.

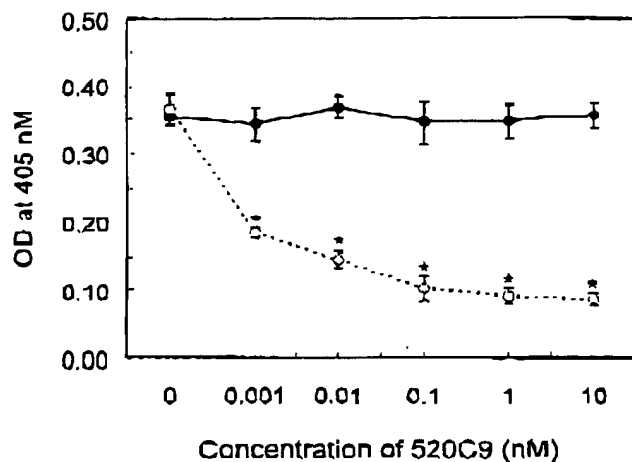


Figure 7 The anti-p185 mAb, 520C9, specifically blocks the binding of H520C9sFv-hIL-2 fusion protein to SKOV 3ip1 cells.

SKOV 3ip1 cells, pre-exposed to serially diluted intact 520C9 monoclonal antibody or to mouse IgG (0.001 to 10 nM), were washed with PBS and incubated with 10 nM H520C9sFv-hIL-2 fusion protein at 37°C for 2 h, as described in the 'Materials and Methods'. Bound fusion protein was measured by indirect ELISA using the anti-human IL-2 polyclonal antibody, EP100. 520C9 mAb (○) caused a dose-dependent decrease in the binding of the fusion protein to SKOV 3ip1 cells. Control mouse IgG (●) had no effect on the binding of the fusion protein. The data are the mean ± s.d. from three separate experiments. * $P < 0.01$ vs normal mouse IgG.

520C9 mAb, it was higher than the K_d observed for the proteolytically cleaved Fab fragment of 520C9 (6.7×10^{-7} M). These findings indicate that humanization of the 520C9 fragment does not dramatically alter its affinity for p185.

The antigen-binding specificity of the H520C9sFv-hIL-2 was determined using indirect ELISA on SKOV 3ip1 cells, which overexpresses p185. As shown in Figure 6a, the conditioned medium from BHK cells transfected with either pcDNA H520C9sFv-hIL-2 or pcDNA H520C9sFv-mhIL-2 displayed binding activity for p185 on the cell surface of SKOV 3ip1 cells. In contrast, the conditioned media containing the normal or mutant H520C9sFv-hIL-2 fusion protein did not bind to HeLa cells (Figure 6b), a result consistent with the fact that these cells do not express p185. As a positive control, the parental 520C9 mAb chemically conjugated to rhIL-2 was shown to bind SKOV 3ip1 cells, but not HeLa cells.

To confirm that the binding of H520C9sFv-hIL-2 fusion protein was specific for p185, SKOV 3ip1 cells were exposed to either the parental 520C9 mAb or normal mouse IgG prior to indirect ELISA. As shown in Figure 7, 520C9 mAb was able to inhibit the binding of the fusion protein to cells in a dose-dependent manner. In contrast, normal mouse IgG did not inhibit the binding of the fusion protein.

Discussion

In this study, we report the construction, expression and characterization of a novel recombinant humanized sFv antibody/hIL-2 fusion protein, H520C9sFv-hIL-2. The humanized sFv of 520C9 was constructed by introducing into a human sFv the amino acids from the complementarity-determining regions of mouse mAb 520C9, that recognize the *HER/neu (c-erbB2)* proto-oncogene product, p185.³⁰ Human IL-2 was then fused to the C-terminus of H520C9sFv by a flexible glycine linker. Following stable expression in BHK cells, this fusion protein was shown to retain both the immunostimulatory activities of IL-2 and the p185 binding characteristics of the parental 520C9 mAb.

Our experiments demonstrate no loss of activity of IL-2 in the H520C9sFv-hIL-2 fusion protein as measured by IL-2-dependent cell proliferation and cytotoxicity assays, compared to rhIL-2 standard. This suggests that the fusion of IL-2 to the C-terminus of the humanized 520C9 sFv does not affect the biological activity of IL-2 and is consistent with previously studies describing the preparation of Fab-IL-2 and SCA-IL-2 fusion proteins.^{31,32} Retention of the IL-2 activity in H520C9sFv-hIL-2 may be partially due to the flexible glycine-rich linker between the C-terminus of sFv and the N-terminus of IL-2 which allows for proper folding of the IL-2 moiety, thereby enabling it to interact with its receptor. This is supported by previous findings with diphtheria toxin-IL-2 fusion proteins demonstrating the importance of the mobility of IL-2 in allowing its interaction to occur effectively.^{33,34}

In antigen-binding ELISAs, EP100 was used to detect the binding activity of H520C9sFv-hIL-2 and H520C9sFv mhIL-2 to cultured SKOV 3ip1 cells overexpressing p185. Our findings demonstrate that these fusion proteins were able to specifically bind p185, and suggest that the IL-2-moiety does not impair its antigen-binding properties. As a positive control, the

parental 520C9 mAb chemically conjugated to hIL-2 also showed antigen-binding activity. The observation that this chemical immunoconjugate has an apparent higher binding affinity to p185, compared to the recombinant fusion proteins, may result from the monomeric antigen binding valency and one IL-2 molecule in the recombinant fusion protein compared to the dimeric antigen binding valency and multiple IL-2 molecules in the chemical immunoconjugate.

Recently an alternative approach to cancer therapy was established by combining effective, high local concentration of IL-2 in the tumor microenvironment and low systemic toxicity.^{7-9,35} This goal was achieved by the construction of a fusion protein consisting of tumor specific mAbs and IL-2. Antibody targeted IL-2 therapy was shown to overcome tumor heterogeneity and eradicate established pulmonary, hepatic and subcutaneous melanoma metastases in a syngeneic tumor model. Tumor lysis was mediated by host immune cells, particularly by specific CD8⁺ T cells. There was also an induction of a long-lived immunity preventing tumor growth in these animals when challenged up to four months later with the same tumor.⁸ In contrast to most other antibody-based therapeutics, antibody-mediated IL-2 delivery can be effective even if only a small percentage of tumor cells are reached, as these are able to elicit a host immune response. Our work may offer a new method of preparing such an important class of therapeutic agents.

Conclusion

In this study we have developed a humanized sFv antibody/human IL-2 fusion protein, which was shown to retain both IL-2 activity and p185 binding affinity. The use of a humanized sFv as the fusion partner over other antibody-based molecules has the potential to reduce the immunogenicity of the fusion protein. In addition, the small size of the sFv fragment would likely improve its pharmacokinetic properties with respect to penetration of solid tumors and clearance. This bifunctional H520C9sFv-hIL-2 fusion protein may thus provide an effective method of targeting therapeutic doses of IL-2 to tumors or other targeted cells without significantly increasing systemic toxicity of IL-2 or its immunogenicity.

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